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PRINCIPAL INVESTIGATOR: Dr. Christopher Sweeney

CONTRACTING ORGANIZATION: Dana Farber Cancer Institute
Boston, MA 02115

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Dr. Christopher Sweeney

E-Mail: christopher_sweeney@dfci.harvard.edu

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14. ABSTRACT

Increasing data is supporting a role for inflammation and more specifically nuclear factor kappa B (NFκB) activation in the development of cancer in general and prostate cancer specifically. More importantly it has been realized that NFκB activation can either promote cancer cell death or cancer cell survival – the outcome being dependent on the context of parallel biological processes. Specifically, the presence of tumor suppressors influence the outcome. In the case of prostate cancer, we will determine whether presence of NFκB activation in the absence of PTEN leads to cancer cell survival and ultimately lethal prostate cancer. Moreover, drugging NFκB activation by blocking IκK (upstream of NFκB) is problematic due to non-NFκB effects. As such this increased understanding of NFκB activation's "context dependency" adds further support for the work we are doing – ie define an NFκB cancer promoting activation signature. In year one of the project we have made significant advances in laying the foundations for developing a risk scoring system based on inflammation/NFκB activation - specifically we have completed the T. Vaginalis and serum protein profiling assays and developed networks of NFκB related genes associated with different biological processes – cell death, migration and proliferation and vasculature development. The current efforts are now focused on correlation with patient outcome.

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INTRODUCTION:
The 4% per year decline in the age specific prostate cancer mortality in the USA has come at the cost of treating a significant number of men who did not require therapy and are living with adverse events that diminish their quality of life. To identify patients with lethal prostate cancer we are deploying a "systems biology approach" to develop a risk scoring system. The systems biology approach is making use of the epidemiological, clinical, pathological and biological data that has implicated Nuclear factor kappa B (NFκB) activation in the development lethal prostate cancer. Specifically, we hypothesize that lethal prostate cancer results from exogenous insults causing NFκB activation which sets up a vicious cycle with further inflammatory insults and culminates in sustained NFκB activation and carcinogenic changes in the microenvironment. This persistent activation results in progression of prostate cancer to a lethal disease. We aim to identify patients with lethal prostate cancer using a systems biology approach focused on the NFκB pathway which will enable the construction of a risk scoring system to identify patients with localized but potentially lethal prostate cancer in need of therapy.

BODY:
New data since the original submission of this grant adds further support to the role for inflammation and more specifically nuclear factor kappa B (NFκB) activation in the development of cancer in general and prostate cancer specifically. More importantly it has been realized that NFκB activation can either promote cancer cell death or cancer cell survival – the outcome being dependent on the context of parallel biological processes. Specifically, the presence of tumor suppressors influence the outcome(1). In the case of prostate cancer, we will determine whether the absence of PTEN in the presence of NFκB activation leads to cancer cell survival and ultimately lethal prostate cancer. Moreover, drugging NFκB activation by blocking IκK (upstream of NFκB) is problematic due to non-NFκB effects(1). As such the increasing knowledge adds further support for the work we are doing – ie find an NFκB cancer promoting activation signature. In year one of the project we have made significant advances in laying the foundations for developing a risk scoring system based on NFκB activation. We have completed the T. Vaginalis and serum protein profiling assays and developed networks of NFκB related genes associated with different biological processes – cell death, migration and proliferation and vasculature development. The current efforts are now focused on correlation with patient outcome.

Task 1. **Identify individual features of NFκB activation which are associated with lethal disease. (Months 1 to 18)**
Task 1A: *Perform gene profiling of tumors and determine whether a set of genes and/or proteins indicative of NFκB activation are associated with lethal prostate cancer. Data will be available at time of commencing the project on 350 patients and we will generate new data on 154 more patients. Data mining and analysis of existing data will be performed to define the 40 gene panel to be assessed for correlation with lethal disease. (Month 1 to 18)*

Accomplishments: In the first 12 months of the grant we have (i) developed putative gene sets from the initial data-mining efforts of publically available data-sets; (ii) been able to establish the "Nugen-Affy" assay for a reliable approach for whole genome expression analysis to be used as the "training set" and (iii) made significant progress in creation of the Tissue Micro-Arrays (TMA) and extract nucleic acids for the "validation set".

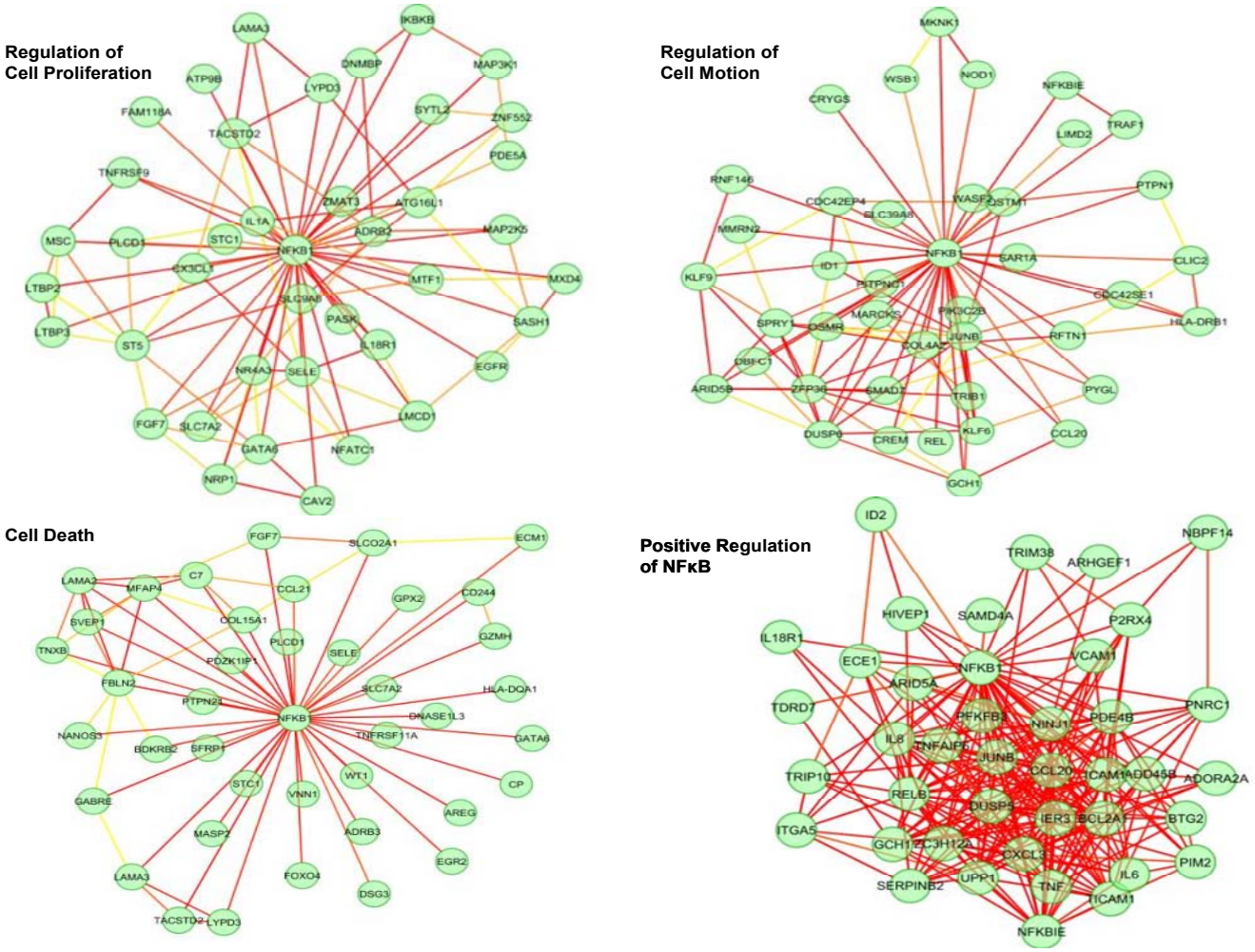
(i) Data-Mining: As detailed in the grant application the research team has mined more than 350 publicly available microarray expression datasets from GEO (2) and ArrayExpress(3) that specifically investigate prostate cancer. They have also performed bioinformatic analyses of an additional >1,500 cancer-specific arrays from these repositories, and non-condition-specific genomic data such as physical and genetic interactions from BioGRID(4) and IntAct (5), transcriptional regulatory relationships from Transfac(6) and cisRED(7), and miRNA data from miRBase(8). These resources have provided literally billions of datapoints which are being integrated with our prostate cancer-specific clinical and genomic data, using methodologies developed by members of our research team(9-12). Such data is key both for enriching detailed mechanistic models of prostate cancer development at the molecular level and, as has been done previously for genetic data(13, 14)

differentiating common functional variation in the general population from causal variation specific to lethal prostate cancer.

Using this approach we have already identified candidate genes: Specifically, we have focussed on transcripts (i) correlated with NFκB activation in external expression data from GEO and ArrayExpress and (ii) targets linked to these genes by regulatory or physical interactions (e.g. EZH2, DAB2IP). Similar systems approaches have been highly successful in illuminating the entirety of the biomolecular pathways contributing to basic biology phenotypes in model organisms (15, 16). We contend that this integrative modeling is critical to understanding and detecting the development of lethal prostate cancer and will define a critical set of genes ("gene-panel") indicative of NFκB activation and in turn lethal cancer.

The second step in this process was to define NFκB networks that correspond to distinct biological processes. We chose to develop 11 networks from a total of 442 different biological or biochemical processes. The key determinant was whether the biological context was related to cancer biology and associated with the hallmarks of cancer and in turn lethal prostate cancer or NFκB related biology. The 11 context analyzed were (i) cell death; (ii) cell migration; (iii) cytokine metabolic process; (iv) mesencyhmal cell differentiation; (v) positive regulation of NFκB, (vi) regulation of cell cycle (vii) regulation of cell differentiation; (viii) regulation of cell motion; (ix) regulation of cell proliferation; (x) stem cell maintenance; (xi) vasculature development. Four of the 11 networks are depicted below

Figure 1: Representative NFKB networks



The notable findings from this preliminary work are that there are distinct networks for the unique biological processes. The team then computed for each gene the frequency of co-occurrence among all genes from these

11 networks. It was noted that NFκB1 co-occurs in all 11 context-specific subnetworks (as expected), NFκBIE in 5, CCL20 in 4. Besides NFκB1 there is no other gene that co-occurs in all 11 context-specific sub-networks.

Our next step is to now assess these gene-sets from these networks with 33 publically available prostate cancer genomic data-sets which we have already curated. This will allow us to "probe" the data-sets and determine which gene-sets are associated with poor clinical outcomes. We will be able to continually assess different putative gene-sets in an iterative manner and keep refining until we are confident we have a robust gene-set to explore further. We will use the following guiding principles

- For all scenarios, we will look at the proposed signature with and without PTEN (ie the major tumor suppressor gene for prostate cancer. As required we will also later see the effect of p53, EZH2 and DAB2IP status. It is anticipated the "cancer promoting NFκB activation signature" will be one that includes loss of a tumor suppressor.
- Use a crude approach and look at the outcome of the genes which are in 5 or more, 4 or more or 3 or more networks. This crude approach makes use of the notion that a set of genes which overlaps multiple biological processes are driving (or at least represent) a number of the hallmarks of cancer and in so doing will indicate a cancer that is primed for rapid proliferation and metastasis and hence lethal prostate cancer.
- Look at gene-sets from the networks which represent "contexts"/biological processes individually with and without PTEN (and other tumor suppressors). For example we will assess the gene-sets from following netoworks
    - cell death
    - cell migration
    - regulation of cell cycle
    - regulation of cell proliferation
    - vasculature development
    - positive regulation of NFκB transcription factor
- Start building on the individual networks based on the leads from outcomes of the individual contexts
    - cell death plus proliferation
    - cell death plus proliferation plus migration

  This will allow an assessment of whether two or more key co-activated biological processes are critical for "lethal" prostate cancer and compound the effect of the individual network.

(ii) Assessment of candidate genes in Training Set: In the original application, the Harvard School of Public Health collaborators had planned to generate gene-expression profile data using the 24,000 DASL platform from prostatectomy specimens from the Health Professionals Follow-up Study and the Physicians Health Study (to be referred to as HPFS/PHS cohort). However, at time of commencing the assays it became apparent the quality of the assay had declined and failed our pilot study. To address this concern, the HSPH have since successfully established the "Nugen-Affy" platform. This work was not being paid for by this DOD contract and there was no change to the budget or scope of work. The change will however result in access to high quality data and ensure we adhere to the original SOW. Specifically, to conduct transcript profiling in FFPE prostate cancer tissues, whole transcriptome amplification is being paired with microarray technologies. Briefly, RNA extracted from FFPE prostate cancer samples is being amplified using the WT-Ovation FFPE System V2 (Nugen, San Carlos, CA), a whole transcriptome amplification system that allows for complete gene expression analysis from archived FFPE samples known to harbor small and degraded RNA. Using a combination 5 and random primer, reverse transcription creates a cDNA/mRNA hybrid. The mRNA is subsequently fragmented, creating binding sites for DNA polymerase. Isothermal strand-displacement, using a proprietary DNA/RNA chimeric SPIA primer, then amplifies the cDNA. To prepare the amplified DNA for microarray hybridization, the cDNA is fragmented and then labeled with a terminal deoxynucleotidyl transferase that is covalently linked to biotin. The labeled cDNA is then hybridized to a GeneChip Human Exon 1.0 ST microarray (Affymetrics, Santa Clara, CA). This array contains roughly four probes per exon and roughly 40 probes per gene, assessing the expression of roughly 28,000 unique genes. **We have recently completed a pilot study using 11-21 year old prostate tumor specimens and found excellent (r > 0.95) concordance of technical replicates and no**

influence of block age on expression profiles. 420 samples will be assayed and 140 patients will have lethal disease post prostatectomy and 280 will be long term survivors/non-lethal outcome. The samples were aliquoted the week of May 21 and data will be available Aug 2012. This time "dove-tails" well with the data from the data-mining (above).

Generation of Discovery Set and Training Sets: Using the genes associated with lethal prostate from the data-mining efforts described above (discovery set) we will then assess them and develop a training set from the genomic "Nugen-Affy" data using SAM and limma and validated both computationally and by testing for enrichment for known biology (using MSigDB/GeneSigDB where appropriate). If necessary, additional QC filtering will be applied and the genes re-queried until convergence on a consistent set of markers reproducibly predictive of lethal prostate cancer. We hope that standard sparse regression (lasso) or feature select will narrow this without loss of predictive accuracy to ~20 genes for application directly to the validation set (Gelb Center samples described below). Our goal is to identify a gene profile with a number of genes which will be feasible for clinical deployment from formalin fixed tissue. The 21 gene profile from Oncotype Dx has shown that such gene sets have both discriminatory power to predict response to adjuvant chemotherapy for breast cancer and can be implemented in the clinic.

The gene-set chosen from the "Nugen-Affy" work will then be subjected to a multivariate analysis and we will determine whether this gene-set/biological variable has a strong enough association with the lethal prostate cancer to be taken to the validation set. We will estimate Cox proportional hazards model with hazard ratios (HR) and the corresponding 95% confidence intervals (CIs) using, both without (unadjusted) or with (adjusted) the adjustment of factors known to be associated with lethal prostate cancer. The variables will include standard criteria – PSA level, Gleason score and pathological stage.

(iii) Validation Sets: In parallel with the above work we have been creating a unique cohort to validate whether the outcomes associated with training set can be reproduced in an independent data-set.

Status of TMA Creation and Extraction of Nucleic Acids from Independent Cohort: Currently we have identified in the DFCI data-base (ie Gelb Center) at least 100 patients with metastases post prostatectomy (lethal) and 230 patients without metastases post-prostatectomy (non-lethal/long-term survivors). When we perform the analysis we will use definitions that are harmonized with the HPFS/PHS cohort. Currently, 3 of the 4 TMAs have been created with each TMA laid out with 3 cores of tumor and 2 cores of benign/normal for each case. The TMAs are being made in duplicate and then cores of tumor and of non-cancer tissue are being obtained (when tissue remains) for nucleic acid studies.

We will apply the signature score of cancer promoting NFκB activation directly to the validation set of 77 lethal:77 non-lethal samples. We have previously calculated a C-statistic for Gleason score of 0.86. Compared to a model with Gleason alone, we estimate that we will have 80% power and type 1 error of 0.05 to detect an improvement in the ROC curve of 6% for the signature of activation (i.e increase to 92%) with a rank correlation between models for both lethal and indolent of 0.8. A multivariate analysis will be used in determining whether a biological variable has a strong enough association with the lethal prostate cancer. We will estimate Cox proportional hazards model with hazard ratios (HR) and the corresponding 95% confidence intervals (CIs) using, both without (unadjusted) or with (adjusted) the adjustment of factors known to be associated with lethal prostate cancer. The variables will include standard criteria – PSA level, Gleason score and pathological stage. The gene-set that meets this criteria will be chosen for the risk assessment tool to be assessed in the biopsy cohorts in Year 3 (Aim 3).

It is of note that we will also be choosing the 4 most predictive genes which have robust antibodies available for immunohistochemistry studies. We will also make use of Nanostring's nCounter platform as detailed in the response to the reviewers' comments at time of grant approval. Use of the TMA and the multiplex assay may allow us to have greater power and analyze more of 100 "lethal" and 230 "non-lethal cases" to have greater power with the same amount of expense. This will depend upon the number of genes chosen.

Task 1B: Perform protein profiling of circulating blood proteins and determine whether a protein or set of proteins indicative of NFκB activation are associated with lethal prostate cancer. Circulating proteins will be assessed in two cohorts of 312 patients. Samples have already been assembled and tied to clinical outcomes. (Month 1 to 18).

We have performed the following assays in 496 patients GROα (CXCL-1), IL-1α, IL-1β, IL-2, IL-6, IL-8, MCP-1 (CCL-2) and TNFα. The assay results and clinical data are currently being analyzed by the statisticians.

	GROα pg/ml	IL-1α pg/ml	IL-1β pg/ml	IL-2 pg/ml	IL-6 pg/ml	IL-8 pg/ml	MCP-1 pg/ml	TNF-α pg/ml
Mean	111.16	0.90	1.06	19.99	8.99	4.79	261.27	5.01
Median	83.90	0.09	0.69	14.06	5.19	3.85	243.72	4.02
Max	994.29	15.30	18.28	270.59	320.41	31.01	1957.95	113.27
Min	0.00	0.00	0.00	0.00	0.00	0.11	1.99	0.00

As described in the submission, we are analyzing the EDRN cohort who had samples at time of prostate biopsy to assess for cancer. We indentified 153 of the planned 153 patients with clinically significant cancer (per Epstein criteria at time of biopsy) and indentified 100 of the 153 proposed patients with clinically indolent cancer (per Epstein criteria at time of biopsy). We have complemented this by also looking at 50 patients with no cancer on biopsy with the goals of assessing whether the no-cancer and clinically indolent patients have a similar profile to each other yet distinct from the patients with more aggressive cancer.

As described in the submission, we are also analyzing the Gelb Center cohort. At the time of submission we planned on assessing 153 patients in the Gelb Center who had a prostatectomy and relapsed but only 84 patients had suitable serum for analysis. In addition only 107 of the 153 non-lethal patients had enough serum for analysis. Given this did not significantly decrease the labor and mutiplex assay costs, no savings were incurred.

The statisticians will assess which one or more inflammatory protein(s) is associated with more aggressive/lethal prostate cancer. A multivariate analysis will be used in determining whether a biological variable has a strong enough association with the lethal prostate cancer. We will estimate Cox proportional hazards model with hazard ratios (HR) and the corresponding 95% confidence intervals (CIs) using, both without (unadjusted) or with (adjusted) the adjustment of factors known to be associated with lethal prostate cancer. The variables will include standard criteria – PSA level, Gleason score and stage (clinical stage if TRUS biopsy cohort and pathological stage if prior to prostatectomy). We anticipate having this work completed in July 2012.

Task 1C: Assess whether seropositivity for Trichimonas vaginalis correlates with NFκB activation and lethal prostate cancer. We will assess the seropositivity from 111 patients with non-lethal and 111 patients with lethal prostate cancer. Samples have already been obtained and correlated with clinical outcomes. (Months 1 to 18)

Data is available and in the hands of the statisticians for analysis. It is of note the Gelb Center samples were done in conjunction with the DOD but funded using institutional funds. This allows for us to extend the analysis to allow greater power and assessment of serostatus across more prostate cancer disease states. The cohorts with an * are extra samples being analyzed with non-DOD funds but supportive for this grant. There sero-prevalence was 13.5% for the entire cohort of 563 patients. We will be able to analyze within cohorts (EDRN – clinically indolent localized vs clinically localized but significant) and Gelb Center (no-relapse post-RP versus relapse post RP) and across the clinical spectrum of low grade low volume to metastatic disease.

- *100 pts with indolent disease at biopsy (EDRN) and 50 patients with elevated PSA and no cancer (was to be 111 patients with indolent disease at biopsy)*
- **111 pts with clinically significant localized disease (EDRN) (as planned)*
- ** 84 Gelb Center prostatectomy not relapsed (extra)*
- ** 107 Gelb Center relapsed post prostatectomy (extra)*
- *111 pts with metastatic disease (ECOG) (as planned)*

A multivariate analysis will be used in determining whether the T. Vaginalis serostatus is a biological variable associated with lethal prostate cancer. We will estimate Cox proportional hazards model with hazard ratios (HR) and the corresponding 95% confidence intervals (CIs) using, both without (unadjusted) or with (adjusted) the adjustment of factors known to be associated with lethal prostate cancer. The variables will

include standard criteria for those with localized disease at time of obtaining the sample – PSA level, Gleason score and stage (clinical stage if TRUS biopsy cohort and pathological stage if post-prostatectomy). We anticipate having this analysis completed in July 2012.

It is also of note that by having the data-sets from patients with localized disease at time of sample acquisition in Tasks 1A and 1B overlapping, we will be able to also determine if sero-positive T.Vaginalis status is also associated with elevation of one or more inflammatory proteins.

Task 1D: Assess whether gene variants associated with NFκB activation are associated with lethal prostate cancer. We will mine existing data sets to define the panel of gene variants to be correlated with lethal disease and then analyze 306 patients. The samples and clinical outcomes have already been assembled. (Months 1 to 18).

Samples have been pulled and DNA isolated from the EDRN samples. Of the 153 samples with clinically indolent disease, we were only able to access 100 patients and supplemented this with 50 patients without cancer. This will allow a "pilot" assessment to assess whether any SNPs noted to be differentially expressed in the high risk/metastatic extreme state of this disease are also not found in patients without cancer. We are in the process of extracting the DNA from the whole blood from the ECOG samples.

At this time, completion of this aim is contingent upon identifying the genes to be assessed. Namely, the genes will be determined from results of the work done in Tasks 1A and 1B as well as published literature on inflammation/NFκB related genes associated with prostate cancer risk(17) and prostate cancer death(18). This will increase the pre-test probability of any SNPs associated with this work will be relevant.

We anticipate performing the SNP analyses in July 2012 and having the data analyzed Aug 2012 (month 18 of the grant). This will provide the data in time for the creation of the risk score in Months 18 to 24 as detailed in the original statement of work. The HSPH GWAS data-base is being prepared for analysis at time of writing the grant so it can be mined as soon as the genes are identified from the results of Tasks 1A and 1B and the literature.

Also of note in a parallel/complementary effort, we will be able to determine whether the germ-line SNPs associated with lethal outcome are also associated with altered gene-expression in the tumor. This work will require funding for SNP assays independent of this DOD grant. However, the project will be feasible as we will have germ-line DNA and corresponding tumor gene expression profile data on about 210 patients with lethal disease and 510 patients who are long term survivors post prostatectomy (combined HSPH and Gelb center cohorts).

KEY RESEARCH ACCOMPLISHMENTS:

- Development of NFκB networks associated with different biological
- Curated publically available prostate cancer data-bases for interrogation
- Developed "Nuge-Affy" assay at HSPH
- Created informative TMAs and nucleic acid resources for validation of work from HSPH cohorts
- Created serum profile for interrogation with prostate cancer outcome – T. vaginalis status and inflammation related proteins.
- Laid the foundation for months 12 to 18 of the grant to generate "new knowledge" relevant to lethal prostate cancer by analyzing the informative data-sets.

REPORTABLE OUTCOMES:

- manuscripts, abstracts, presentations: None to date but data should be available in the next 6 months
- licenses applied for and/or issued: None
- degrees obtained that are supported by this award; None
- development of cell lines, tissue or serum repositories: Creation TMA and nucleic acid collection annotated with clinical outcome in collaboration with Gelb Center
- informatics such as databases and animal models, etc.: None
- funding applied for based on work supported by this award: None
- employment or research opportunities applied for and/or received based on experience/training supported by this award: None

CONCLUSION:

As detailed above, we have generated a significant amount of data and are now ready to perform robust statistical analyses which will lead to reliable new findings. We anticipate having findings either supporting or refuting the hypothesis that markers of inflammation and/or NFκB activation is associated with lethal prostate cancer in 6 months. This data will be able to tell us whether serum protein profiling and/or germline SNP and/or tumor gene expression profiling focused on inflammation can be used as a prognostic factor in patients diagnosed with prostate cancer. Moreover, it will set the stage for applying these findings to assess whether one or more of the findings can identify patients with clinically localized disease and suitable for surveillance as well identify a biology (inflammation) or target (NFκB) to abrogate and prevent progression on surveillance and/or eradication of micrometastatic disease post definitive local therapy.

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APPENDICES:
None

SUPPORTING DATA:
None